INACTIVATION OF TWO ARBOVIRUSES AND THEIR ASSOCIATED INFECTIOUS NUCLEIC ACIDS

LEONARD A. MIKA, JULIUS E. OFFICER AND ARTHUR BROWN

Tl
iltir
chan
ion
depe
and
or m
activ
occu
tious
have
and
the

A
ied
and/
(Bac
1960
Gin
1960
1961
heat
follo
tion
biph
tion
prot
age t
ing h
that
tulat
acid
ing r
studi

Eff
anim
Polla
Rush
and

Rec

INACTIVATION OF TWO ARBOVIRUSES AND THEIR ASSOCIATED INFECTIOUS NUCLEIC ACIDS

LEONARD A. MIKA, JULIUS E. OFFICER AND ARTHUR BROWN
From the U. S. Army Biological Laboratories, Fort Detrick, Frederick, Maryland

The complete inactivation of a virus ultimately involves an irreversible change in its nucleic acid. The expression of viral inactivation, however, is dependent on the whole virus structure and is frequently observed as a two-step or multi-step alteration of the biological activity before complete inactivation occurs. Techniques for isolating infectious nucleic acids from certain viruses have stimulated studies of the physical and chemical inactivation in relation to the basic infectious unit of the virus.

A number of investigators have studied the thermal inactivation of viruses and/or their infectious ribonucleic acids (Bachrach, 1959, 1961; Bachrach et al, 1960; Friedman and De Berry, 1959; Ginoza, 1958; Norman and Veomett, 1960; Papaevangelou and Youngner, 1961; Youngner, 1957). Although the heat inactivation of some animal viruses follows first order kinetics, the inactivation of others has been identified as a biphasic reaction. This latter inactivation is thought to be the result of initial protein denaturation followed by damage to the viral nucleic acid. As a unifying hypothesis, Woese (1960) suggested that primary damage to either of 2 postulated interconvertible forms of nucleic acid could resolve the apparent conflicting results obtained in heat inactivation studies.

Effects of ultraviolet irradiation on animal viruses have been reviewed by Pollard (1953), Kleczkowski (1960), Rushizky et al (1960), Taylor (1960), and Wasserman (1962). These investigators found that the lethal effect of such irradiation upon viruses was usually a first order reaction and due primarily to the absorption of the ultraviolet photons within the nucleic acid or nucleoprotein.

Nitrous acid appeared to be an effective inactivating and mutagenic agent for a number of viruses and their associated nucleic acids (Schuster and Schramm, 1958; Mundry and Gierer, 1958; Boeyé, 1959; Granoff, 1961; Wasserman, 1962). This inactivation may follow either monophasic or biphasic kinetics. Deamination of protein and/or nucleic acid appeared to be the basis for the results ontained. Thus, while inactivation of tobacco mosaic virus (TMV) and its extracted nucleic acid proceeds at the same rate, poliomyelitis virus is inactivated at a faster rate than its extracted nucleic acid. This suggests that, with poliomyelitis virus, damage to the surface protein component occurs first and continues at a faster rate than that to the extracted nucleic acid.

The present investigations were initiated to define and to compare the responses of 2 lipid-containing viruses, the etiological agents of Eastern and Venezuelan equine encephalitis (EEE and VEE), to thermal, ultraviolet light, and nitrous acid (HNO_2) inactivation. Emphasis was placed on describing the processes of viral inactivation in relation to the basic infectious unit of the virus, the ribonucleic acid (RNA).

METHODS

Viruses.—The Trinidad strain of VEE virus (Randall and Mills, 1944)

Received for publication June 12, 1963.

and the Louisiana strain of EEE virus (Howitt et al, 1948) were employed in these investigations. Ten-day-old chick embryos were harvested 18 to 22 hours after infection via the allantoic route with approximately 10^5 plaque-forming units (PFU) of virus per ml. Each viral seed consisted of 10% chick embryo suspension in either heart infusion broth or 0.02 M sodium phosphate buffer (PB), pH 7.4. After homogenization, the suspensions were clarified by 2 consecutive low speed centrifugations at 350 rcf for 10 minutes. Partially purified virus in PB was prepared for the heat inactivation studies by differential centrifugation. Centrifugation at 1500 rcf for 15 minutes was followed by centrifugation of the supernatant fluid at 135,000 rcf for 120 minutes. Pellets were resuspended in a reduced volume of PB (25-fold concentration), and the suspension was subjected to another low speed centrifugation.

Titration of infectivity.—The viruses and their associated RNA's were assayed by counting plaques in chick fibroblast (CF) cells. Monolayers of CF cells were grown in 60-mm Petri dishes with a medium composed of 0.5% lactalbumin hydrolyzate, 89.5% Hanks' balanced salt solution, and 10% calf serum. Serial 10-fold dilutions of the viruses were made in phosphate-buffered saline (PBS). Each plate was inoculated with 0.1 ml. Adsorption was then carried out for 30 minutes at room temperature. Infectious RNA's were titrated by first washing monolayer sheets of CF cells once with PB and then with 0.5 M NaCl contained in the same buffer. Serial 10-fold dilutions of the nucleic acid preparations were made in 1 M NaCl, and 0.1 ml of the inoculum was added to each plate. Adsorption was allowed to proceed at room temperature for 20 to 25 minutes.

Cultures were overlaid with 5 ml Hanks' balanced salt solution containing 0.5% lactalbumin hydrolyzate, 0.1% yeast extract, 0.5% gelatin, 0.14% $NaHCO_3$, and 1.1% agar. The cell monolayers were stained at 48 hours by adding either 1.5 ml of the overlay medium containing a 1:10,000 dilution of neutral red or 1.5 ml of aqueous neutral red (1:5000).

Extraction of RNA with phenol.—The infectious RNA fractions were extracted from partially purified virus or from infected 10% chick embryo suspensions by the hot extraction procedure of Wecker (1959). Viral samples were treated with an equal volume of molten phenol and shaken at 50 C for 5 minutes. The phases were separated by centrifugation at 1500 rcf for 15 minutes at 4 C. The aqueous phase was collected and then readjusted to its original volume with PB. The entire extraction procedure was repeated twice. The nucleic acid was then precipitated from the aqueous phase with 67% ethanol and/or 1 M NaCl at 4 C and centrifuged at 350 rcf. The RNA pellets were dissolved in PB for assay or inactivation studies. The titers of the RNA preparations, in terms of the original viral titers, ranged from 0.01 to 0.1% for VEE and 0.03 to 0.7% for EEE.

Treatment of the RNA samples with 1 to 5 μg per ml of ribonuclease for 10 minutes at 37 C completely destroyed infectivity; treatment with deoxyribonuclease did not. Identical enzymatic treatment of the viral suspensions had no effect. The extracts had an ultraviolet spectrum characteristic of nucleic acid, showing an absorption maximum at 258 mμ and a minimum at 232 mμ. The average ratio E_{258}/E_{230} from a series of samples was 1.90. Such tests characterized the extracts as typical RNA.

Recoverable RNA is defined as the infectious RNA extracted from a viral suspension after the suspension was

physically or chemically inactivated. In contrast, extracted RNA defines the nucleic acid extracted with phenol from an untreated viral suspension. The extracted nucleic acid was later subjected to physical or chemical inactivation.

Heat inactivation.—Thin walled, 5-ml ampuls containing either 1 ml of a viral suspension or 0.8 ml of extracted RNA were sealed and placed in a water bath at 50 C. At selected intervals of time, ampuls were removed and their contents immediately chilled and assayed. Concurrently, the infectivity of the recoverable RNA from heated virus was determined.

Ultraviolet irradiation.—Viral seeds were diluted 1:10 or 1:100 in PBS, and 1.5 ml were added to a number of 100-mm Petri dishes. Because of the limited amount of extracted RNA available, only 0.4 ml was added to 60-mm dishes to provide a comparable depth for irradiation. The preparations were irradiated under a 15-watt General Electric germicidal lamp at a distance of 25 cm with constant agitation. The lamp intensity at 2537 A was 271 μwatts per cm^2 at this distance. Immediately after exposure, decimal dilutions of the suspensions were made for titration.

Inactivation by nitrous acid.—A modification of the method of Mundry and Gierer (1958) was employed for inactivation by nitrous acid. One ml each of 1 M sodium acetate buffer, pH 4.5, and 4 M sodium nitrite were mixed either with 2 ml of virus (a 1:10 or 1:100 dilution in 0.01 M PB of a 10% suspension of infected chick embryo) or with the extracted nucleic acid in the same phosphate buffer. The inactivation rates of viral suspensions by HNO_2 were first compared at 4, 22, 37 and 50 C. Later, all inactivation studies were carried out at 22 C. To stop the action of HNO_2, portions of the mixture were placed in an ice water bath and diluted 1:10 with 0.1 M PB at pH 7.5. Alternatively, the pH was adjusted to pH 7.5 with NaOH, and assays were performed immediately.

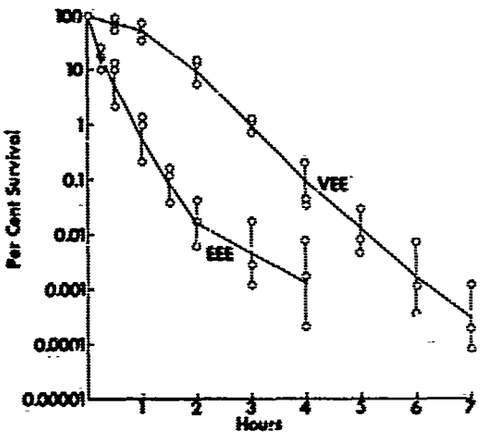

FIGURE 1.—Inactivation of Eastern and Venezuelan equine encephalitic viruses at 50 C.

RESULTS

Heat inactivation.—When thermal inactivation was carried out at 50 C with infected chick embryo homogenates, EEE and VEE viruses were inactivated at different rates depending on the dilution of the virus used as starting material. These differences were accentuated when greater dilutions of the homogenates were heated. This suggested that extraneous material in the preparations offered some protection against heat inactivation. To minimize this protection and to demonstrate differences between the 2 viruses, diluted, partially purified virus was used as starting material. Figure 1 shows the kinetics of inactivation of the 2 partially purified viral suspensions in 3 separate experiments. Although the initial viral concentrations were similar, the titer of VEE virus during thermal inactivation approached the lower limits of the assay method within 7 hours, while this occurred within 4 hours with EEE virus. The curve for EEE virus appeared to be biphasic, the 1st phase being steeper in

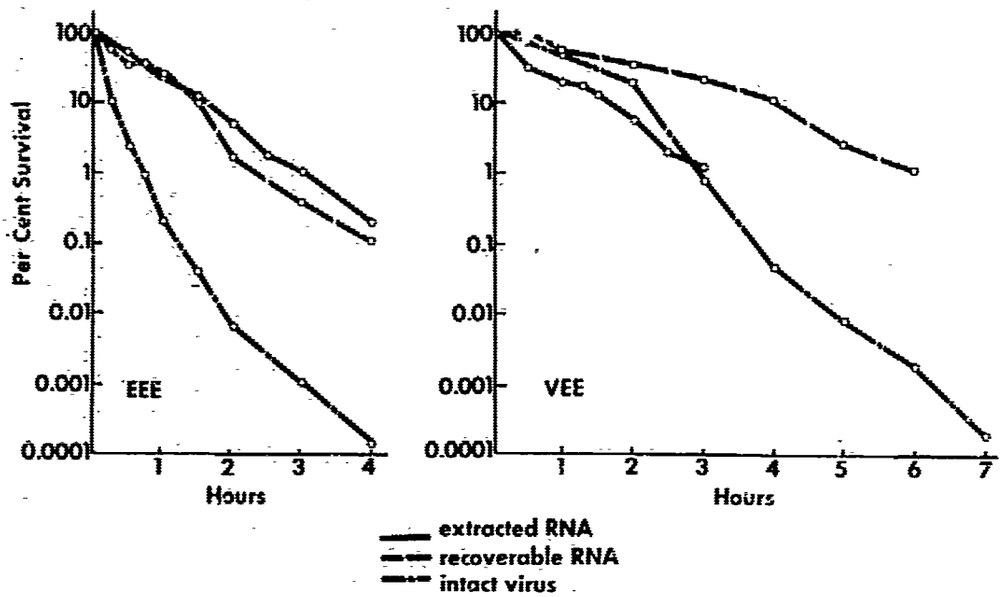

FIGURE 2.—Comparison of inactivation rates at 50 C of Eastern and Venezuelan equine encephalitic viruses and their associated nucleic acids.

slope in the first 2 hours. This was similar to the results reported for other viruses. Although the inactivation of VEE virus also appeared biphasic, the phases were reversed as compared with those exhibited by EEE virus.

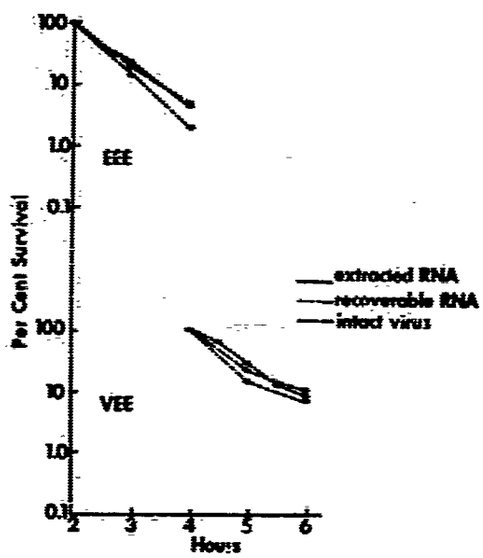

FIGURE 3.—Terminal phases in the heat inactivation of Eastern and Venezuelan equine encephalitic viruses and their associated nucleic acids.

Thermal inactivation of virus, recoverable RNA, and extracted RNA.—After examining the inactivation curves of the viruses and the RNA recoverable from these viruses (figure 2), one might conclude that the recoverable RNA's vary a great deal in their heat resistance and that this could explain the difference in heat stability of the respective viruses. The difference in the recoverable RNA curves was more likely due to the more rapid alteration of the lipoprotein component of one of the viruses. Accordingly, differences in recoverable RNA curves reflected differences in the protective capacity of the lipoproteins. For each virus it appeared that heat inactivation was first directed at the lipoprotein coat, since the loss in activity in the early stages was greater for the virus than for its recoverable RNA. In contrast to the inactivation curves of each intact virus and its recoverable RNA, the extracted RNA's from each virus

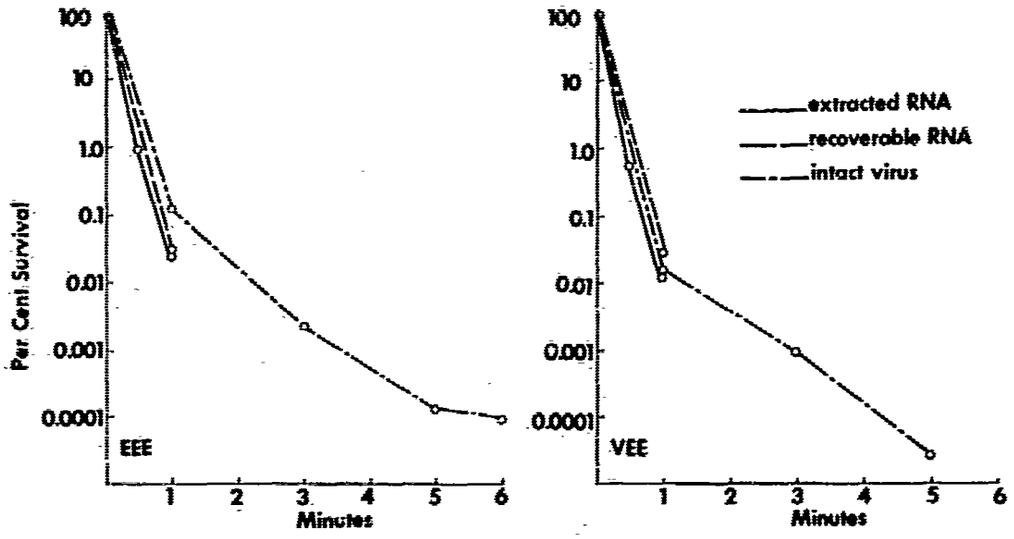

FIGURE 4.—Ultraviolet inactivation of Eastern and Venezuelan equine encephalitic viruses and their associated nucleic acids.

could not be differentiated by their resistance to heat.

Figure 3 illustrates the similarity of the terminal portions of the inactivation curves of each virus, the recoverable RNA, and the extracted RNA. It is concluded from these data that in the terminal phase the heat inactivation of the virus is largely determined by its hitherto protected basic infectious unit, the RNA.

Inactivation of virus, recoverable RNA, and extracted RNA by ultraviolet light.—Although the plaque titer of VEE virus was below the limits of assay after only 6 minutes exposure, 10 PFU per ml of EEE virus were detectable after an equal time exposure (figure 4). The survival curves of both viruses were parallel, and both exhibited 2 components. Infectious RNA was not recovered from either virus after irradiation for 3 minutes. The data also indicate that the extracted RNA's from both viruses were inactivated at similar rates to a loss of 3 logs within 1 minute of exposure. After 2 minutes of irradiation the titer of the extracted RNA was below the limits of assay.

The effect of this irradiation appears to be directed specifically at the nucleic acid component, as evidenced by the similar rapid inactivation of virus, recoverable RNA, and extracted RNA. Limitations of RNA extraction procedures and assay techniques obscured the RNA inactivation (both recoverable and extracted) during the 2nd phase of the virus inactivation. Additional studies are necessary for a complete analysis of the mechanism of ultraviolet inactivation, especially since a biphasic inactivation curve for the viruses was obtained.

Nitrous acid inactivation.—The action of HNO_2 on the arboviruses is temperature-dependent (table 1). Both EEE VEE viruses were inactivated more slowly at 4 C than at higher temperatures. In fact, virus was detectable after 20 minutes exposure to HNO_2 in the cold, although virus could not be detected after 30 minutes treatment. At room temperature the rate of inactivation after 1 and 2 minutes was comparable to that obtained after 10 and 20 min-

TABLE 1.—Inactivation of Eastern and Venezuelan equine encephalitic viruses by nitrous acid.

Virus	Temperature, C	Time of exposure, minutes							
		0*	1	2	5	10	20	30	40
EEE	4	6.7†				4.7	3.9	<2.0	
	22	6.7	5.1	3.8	<2.0				
	37	6.7	<2.0						
	50	6.7	<2.0						
VEE	4	7.1				3.9	2.7	<2.0	<2.0
	22	7.1	3.7	2.9	<2.0				
	37	7.1	<2.0						
	50	7.1	<2.0						

* Titrated immediately after addition of nitrous acid.
† Log plaque-forming units per ml.

utes at 4 C. The inactivations of both viruses at 37 and 50 C were too rapid to measure. In general, the data indicate that VEE virus was inactivated somewhat more rapidly by HNO_2 than was EEE virus. These results were opposite to those of the experiments on thermal inactivation. They suggest, however, that HNO_2 treatment, like heat, might act first or more rapidly on the lipoprotein fraction of the virus than on the nucleic acid component. To test this premise, an attempt was made to recover RNA from HNO_2-treated virus and to treat extracted RNA from infected embryos with HNO_2. The findings (figure 5) indicate clearly that RNA can be recovered easily after inactivation of both viruses with HNO_2. Infectious virus was barely detectable after a 2-minute exposure to HNO_2, although the infectivity of recoverable RNA was demonstrable even after 10 minutes of treatment. The extracted RNA was also inactivated slowly by the HNO_2 treatment, and infectivity could still be detected after 10 minutes of inactivation.

DISCUSSION

The extraction of infectious nucleic acids from certain viruses has provided a valuable tool with which to examine the mechanism of viral inactivation in terms of its basic infectious (and genetic) unit. The results described herein, showing differences in the rate of heat inactivation between EEE and VEE viruses, may best be interpreted as

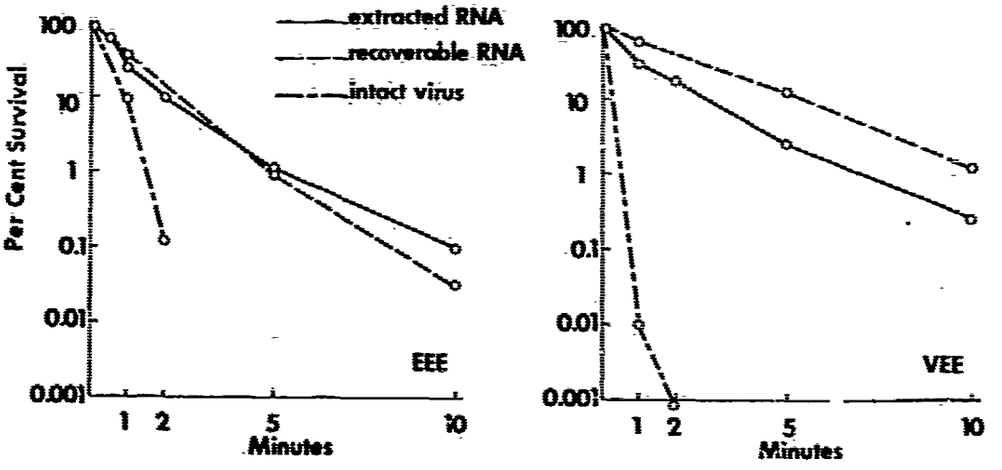

FIGURE 5.—Nitrous acid inactivation (22 C) of Eastern and Venezuelan equine encephalitic viruses and their associated nucleic acids.

revealing differences in their surface (lipoprotein) components, since their extracted nucleic acids were inactivated at the same rate. The same reasoning suggests that the difference in inactivation rates of the recoverable RNA curves from the 2 viruses could be explained by the different capacities of surface constituents to protect the RNA. Ancillary data support the concept that significant differences exist in surface composition (e.g., specific neutralizing antigens) of the 2 viruses.

With both viruses there appeared to be a biphasic or a gradual inactivation curve. Such curves have been interpreted by others (Wasserman, 1962; Boeyé, 1959) as showing the presence of at least 2 components.

Woese (1960) suggested that the 2 components might correspond to different interconvertible forms of viral nucleic acid, that primary damage at any temperature is imposed upon the nucleic acid, and that protein was subsequently denatured when it was left unprotected by the denatured nucleic acid. From our data it appears unlikely that the nucleic acid was inactivated first, because the inactivation curves for EEE and VEE viruses were markedly different, while the inactivation curves of their extracted RNA's were similar. Disregarding the fact that the proposed interconvertible forms of viral nucleic acid have not as yet been reported, one must make complicated assumptions when our data are examined in the light of Woese's hypothesis. It must be assumed that the extraction procedure has selected against one of the postulated interconvertible forms and that the nucleic acid form selected against by the extraction technique differed in heat resistance. This is in contrast to the extracted RNA's which had similar rates of heat inactivation. Our results support more readily a concept of biphasic or multistage inactivation of the different chemical components (i.e., lipoprotein and nucleic acid) of a single viral particle. It is suggested that the last phase most likely represents the inactivation of infectious RNA, or perhaps an intermediate infectious form (Colón and Idoine, 1963). Colón (personal communication) has found that in the terminal phase of inactivation (50 C) the titers of EEE virus were higher in a hypertonic than in an isotonic assay system. The former is more efficient for the detection of infectious nucleic acid (see also Koch, 1960). Although the extracted nucleic acids of the 2 viruses were similar in heat resistance, the heat stability of the surface components differed markedly. The surface coat (lipoprotein) of VEE virus was more resistant to effects of heating than was the surface component of EEE virus. Consequently, the integrity of the nucleic acid structure was presumably protected by preventing heat expansion of the nucleic acid (see Pollard, 1960).

The fact that our heat inactivation curves were not precisely biphasic and represented instead a gradual inactivation may be the result of a gradual loss of different infective properties, e.g., reduced efficiency of adsorption and/or penetration (McLean, 1960).

The inactivation of VEE and EEE viruses by HNO_2 occurred so rapidly that it was impossible to be certain about the nature of the curves. Data obtained with VEE virus do not suggest a linear response but rather a biphasic one. In fact, the gradually changing slopes of the inactivation curves of the extracted and recoverable RNA showed at least 2 components that were potentially involved in the inactivation. There was an indication also that inactivation curves of the extracted RNA from both viruses might be biphasic. This would be in disagreement with the findings of

Schuster and Schramm (1958), Mundry and Gierer (1958), Boeyé (1959), Granoff (1961), and Wasserman (1962). It is presently unknown whether such a disagreement is due to an artifact (e.g., technique) or to gross differences in the properties of the nucleic acids of the different viruses, as Papaevangelou and Youngner (1961) have shown to exist for 2 poliovirus mutants heated at 50 C. In their study the extracted RNA curve was monophasic for one mutant and biphasic for the other.

The recoverable RNA curves were similar to the extracted RNA curves in studies of HNO_2 inactivation. This suggested that Gard's (1960) hypothesis of a "hardened" permeability barrier resulting from the initial stage of a multistage process when formaldehyde denatures the surface protein of poliovirus does not apply to nitrous acid inactivation of the arboviruses. It appeared, rather, that a change in the surface components and the nucleic acids began simultaneously but that the rate of nucleic acid inactivation occurred more slowly. This interpretation is consistent with our data and with the known deamination of both nucleic acids and proteins by nitrous acid. Biphasic curves describing the inactivation rates have been described for some animal viruses (Wasserman, 1962). Monophasic inactivation has been detected in studies with TMV. The latter was attributed to the direct inactivation of the nucleic acid component (Mundry and Gierer, 1958; Schuster and Schramm, 1958). The fact that the RNA of TMV may be partially exposed (Franklin et al. 1957) and that the method of assay of TMV bypasses the role of viral protein in adsorption may account for the monophasic curve.

Ultraviolet irradiation produced biphasic inactivation of each virus. Similar results were obtained by Wasserman (1962) for 2 adenoviruses, although monophasic inactivation curves were obtained with other adenoviruses under similar conditions. Considering the known action of ultraviolet irradiation on nucleic acids, there was no adequate explanation for the biphasic curves obtained by him and others. The present results, including studies on the recoverable and extracted RNA, suggested no explanation of the biphasic curves obtained with the arboviruses. The possibility that multiplicity reactivation could account for the biphasic nature of the curves, as suggested by Luria and Dulbecco (1949) for bacteriophage, by Henle and Liu (1951) for influenza virus, and by Abel (1962) for poxviruses, cannot be profitably explored until good genetic markers for these arboviruses are available.

The results presented in this paper emphasize that differences can be observed in the reaction of 2 closely related viruses after their exposure to heat and nitrous acid, but not to ultraviolet irradiation. Except for ultraviolet irradiation, which presumably inactivated the nucleic acid with minimum interference from other viral components, the biphasic (or multiphasic) curves were an indication that at least 2 components were involved in the inactivation. However, observations reported in the literature and in the present investigations suggest that the nature of the virus, the inactivating agent, the conditions of inactivation, and the method of assay will determine the precise kind of curve that is obtained.

SUMMARY

The inactivation of 2 distinct but related arboviruses (Eastern and Venezuelan equine encephalitis) by heat (50 C), nitrous acid (HNO_2), and ultraviolet light was studied in relation to the infectious ribonucleic acid (RNA). The

2 viruses could be distinguished by their heat inactivation curves. Although the curves for both viruses were approximately biphasic, their phases were reversed. The heat inactivation rates of recoverable RNA (from the heated virus particle) and of extracted RNA (from unheated virus) were less than those for the virus. These results, together with differences between the 2 viruses in the curves of the recoverable RNA and similarities in the inactivation of their extracted RNA, suggested that heat acts first on the surface (lipoprotein) component and then on the nucleic acid. The difference between the 2 viruses in their susceptibility to heat is probably due to differences in their surface lipoprotein components.

The kinetics of inactivation of the 2 viruses and their RNA's by HNO_2 suggested that inactivation of both surface protein and nucleic acid began simultaneously but that the latter inactivation was slower. The respective viruses and their recoverable RNA could be distinguished by their rates of inactivation.

The results with ultraviolet irradiation agree with the concept of primary damage to the nucleic acid. The biphasic nature of the virus inactivation curve was left unexplained. The 2 viruses and their recoverable or extracted RNA's could not be differentiated by their rates of inactivation.

The apparently discrepant results and interpretations of others in regard to monophasic vs. biphasic or gradual inactivation curves were discussed. The precise curves to be obtained will be determined by the nature of the virus and of the inactivating agent, the conditions of inactivation, and the method of virus assay.

REFERENCES

Abel, P. 1962, Virology 17:511–519.
Bachrach, H. L. 1959, Biochem Biophys Res Commun 1:356–360.
Bachrach, H. L. 1961, Proc Soc Exp Biol Med 107:610–613.
Bachrach, H. L., Patty, R. E. and Pledger, R. A. 1960, Proc Soc Exp Biol Med 103:540–542.
Boeyé, A. 1959, Virology 9:691–700.
Colen, J. I. and Ideine, J. B. 1963, Bact Proc, p. 159
Franklin, R. E., Klug, A. and Holmes, K. C. 1957, The nature of viruses. (CIBA Foundation Symposium) London, J & A Churchill, Ltd., pp. 39–55.
Friedman, M. and De Berry, P. 1959, J Immun 82:535–541.
Gard, S. 1960, Ann NY Acad Sci 83:638–648.
Ginoza, W. 1958, Nature 181:958–961.
Granoff, A. 1961, Virology 13:402–408.
Henle, W. and Liu, O. C. 1951, J Exp Med 94:305–322.
Howitt, B. F., Bishop, L. K., Gerrie, R. H., Kissling, R. E., Hauser, G. H. and Trueting, W. L. 1948, Proc Soc Exp Biol Med 68:70–72.
Kleczkowski, A. 1960, Ann NY Acad Sci 83:661–669.
Koch, G. 1960, Virology 12:601–603.
Luria, S. E. and Dulbecco, R. 1949, Genetics 34:93–122.
McLean, I. W. 1960, Ann NY Acad Sci 83:753–760.
Mundry, K. W. and Gierer, A. 1958, Z Vererbungsl 89:614–630.
Norman, A. and Vennett, R. C. 1960, Virology 12:136–139.
Papaevangelou, G. J. and Youngner, J. S. 1961, Virology 15:509–510.
Pollard, E. C. 1953, The physics of viruses. New York, Academic Press.
Pollard, E. C. 1960, Ann NY Acad Sci 83:654–660.
Randall, R. and Mills, J. W. 1944, Science 99:225–226.
Rushizky, G. W., Knight, C. A. and McLaren, A. D. 1960, Virology 12:32–47.
Schuster, H. and Schramm, G. 1958, Z Naturforsch (B) 13:697–704.
Taylor, A. R. 1960, Ann NY Acad Sci 83:670–683.
Wasserman, F. E. 1962, Virology 17:335–341.
Wecker, E. 1959, Virology 7:241–243.
Woese, C. 1960, Ann NY Acad Sci 83:741–751.
Youngner, J. S. 1957, J Immun 78:282–290.

Reprinted from the Journal of Infectious Diseases
November-December, 1963, Vol. 113
Pages 195-203